

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : C12N 15/12, 1/21, A61K 38/38, C12P 21/02, C12N 15/11, 1/20</p>	<p>A1</p>	<p>(11) International Publication Number: WO 95/23857 (43) International Publication Date: 8 September 1995 (08.09.95)</p>
<p>(21) International Application Number: PCT/GB95/00434 (22) International Filing Date: 1 March 1995 (01.03.95) (30) Priority Data: 9404270.2 5 March 1994 (05.03.94) GB (71) Applicant (for all designated States except US): DELTA BIOTECHNOLOGY LIMITED [GB/GB]; Castle Court, Castle Boulevard, Nottingham NG7 1FD (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): KERRY-WILLIAMS, Sean, Martin [GB/GB]; 131 Trent Road, Beeston Rylands, Nottingham NG9 1LP (GB). GILBERT, Sarah, Catherine [GB/GB]; 65 Dene Road, Headington, Oxford OX3 7EQ (GB). (74) Agents: BASSETT, Richard et al.; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).</p>	<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	

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<p>(54) Title: YEAST STRAINS AND MODIFIED ALBUMINS</p>	
<p>(57) Abstract</p> <p>Albumin, for example human albumin, is expressed and secreted in yeast which has been mutated to lack the yeast aspartyl protease 3 (Yap3p) or its equivalent, thereby reducing the production of a 45kD albumin fragment. A further reduction is achieved by additionally deleting the Kex2p function. Alternatively, a modified albumin is prepared which is not susceptible to Yap3p cleavage, for example human albumin which is R410A, K413Q and K414Q.</p>	<div style="text-align: center;"> <p>A B C</p> <p style="text-align: right;">--rHA monomer</p> <p style="text-align: right;">--fragment</p> </div>

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YEAST STRAINS AND MODIFIED ALBUMINS

Field of the invention

- 5 The present invention relates to the production of recombinant human albumin (rHA) by yeast species.

Background and prior art

- 10 Human serum albumin (HSA) is a protein of 585 amino acids that is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The
15 production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

In recent years yeast species have been widely used as a host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992),
20 including rHA (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for production of secreted as well as cytosolic proteins.

- 25 When *S. cerevisiae* is utilised to produce rHA, the major secreted protein is mature 67kDa albumin. However, a 45kDa N-terminal fragment of rHA is also observed (Sleep *et al*, 1990). A similar fragment is obtained when rHA is expressed in *Kluyveromyces* sp. (Fleer *et al*, 1991) and *Pichia pastoris* (EP 510 693). The fragment has the same N-terminal amino acid sequence as
30 mature rHA, but the carboxy terminus is heterogeneous and occurs between

mutagenesis of the codon for Lys⁴¹⁴, resulted in reduction in the amount of the fragment.

5 There is a vast array of yeast proteases which could, in principle, be degrading a desired protein product, including (in *S. cerevisiae*) yscA, yscB, yscY, yscS, other vacuolar proteinases, yscD, yscE, yscF (equivalent to kex2p), ysc α , yscIV, yscG, yscH, yscJ, yscE and kex1.

10 Bourbonnais *et al* (1991) described an *S. cerevisiae* endoprotease activity specific for monobasic sites, an example of which (Arg⁴¹⁰) exists in this region of albumin. This activity was later found to be attributable to yeast aspartyl protease 3 (Yap3) (Bourbonnais *et al*, 1993), an enzyme which was originally described by Egel-Mitani *et al* (1990) as an endoprotease similar to Kex2p in specificity, in that it cleaved at paired basic residues. Further work suggested
15 that Yap3p is able to cleave monobasic sites and between, and C-terminal to, pairs of basic residues, but that cleavage at both types of sites is dependent on the sequence context (Azaryan *et al*, 1993; Cawley *et al*, 1993).

As already discussed, the region of the C-terminus of rHA fragment contains
20 both a monobasic (Arg⁴¹⁰) and a dibasic site (Lys⁴¹³Lys⁴¹⁴). However, even though a Kex2p-like proteolytic activity is present in human cells and is responsible for cleavage of the pro sequence of HSA C-terminal to a pair of arginine residues, the fragment discussed above is not known to be produced in humans. This indicates that the basic residues Arg⁴¹⁰, Lys⁴¹³ and Lys⁴¹⁴ are
25 not recognised by this Kex2p-like protease, in turn suggesting that this region of the molecule may not be accessible to proteases in the secretory pathway. Thus, the Yap3p protease could not have been predicted to be responsible for the production of the 45kDa fragment. In addition, Egel-Mitani *et al* (1990 *Yeast* 6, 127-137) had shown Yap3p to be similar to Kex2p in cleaving the
30 MF α propheromone. Since removal of the Kex2p function alone does not

reduce the amount of the fragment produced, there was no reason to suppose that removal of the Yap3p function would be beneficial. Indeed, Bourbonnais *et al* (1993) showed that *yap3* strains had a decreased ability to process pro-somatostatin, and therefore taught away from using *yap3* strains in the production of heterologous proteins.

Summary of the invention

The solution to the problem identified above is, in accordance with the invention, to avoid or at least reduce production of the fragment in the initial fermentation, rather than to remove it during purification of the albumin. We have now found that, out of the 20 or more yeast proteases which are so far known to exist, it is in fact the Yap3p protease which is largely responsible for the 45kD fragment of rHA produced in yeast. The present invention provides a method for substantially reducing the amount of a 45kDa fragment produced when rHA is secreted from yeast species. The reduction in the amount of fragment both improves recovery of rHA during the purification process, and provides a higher quality of final product. A further, and completely unexpected, benefit of using *yap3* strains of yeast is that they can produce 30-50% more rHA than strains having the Yap3p function. This benefit cannot be accounted for merely by the reduction of rHA fragment from ~15% to 3-5%.

Thus, one aspect of the present invention provides a process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

Preferably, the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.

Suitably, the yeast is *S. cerevisiae* which lacks a functional *YAP3* gene. However, the invention is not limited to the use of *S. cerevisiae*, since the problem of 45 kDa fragment production is found also in other yeast genera, for example *Pichia* and *Kluyveromyces*, which shows that they have equivalent proteases (ie Yap3p proteolytic activity); see Clerc *et al* (1994), page 253. We have confirmed this by hybridisation analysis to locate homologues of Yap3p in non-*Saccharomyces* genera. A gene is regarded as a homologue, in general, if the sequence of the translation product has greater than 50% sequence identity to Yap3p. In non-*Saccharomyces* genera, the Yap3p-like protease and its gene may be named differently, but this does not of course alter their essential nature.

The level of fragment can be reduced still further if, as well as substantially eliminating the Yap3p proteolytic activity, the Kex2p function is also substantially eliminated even though, as mentioned above, elimination of the Kex2p function alone does not affect the level of fragment. As in the case of Yap3p, the Kex2p function is not restricted to *Saccharomyces*; see Gellissen-*et al* (1992), especially the sentence bridging pages 415 and 416, showing that *Pichia* has a Kex2p function. The genes encoding the Kex2p equivalent activity in *Kluyveromyces lactis* and *Yarrowia lipolytica* have been cloned (Tanguy-Rougeau *et al*, 1988; Enderlin & Ogrydziak, 1994).

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease (Rothstein, 1983). Alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations by the process of transplacement (Winston *et al*, 1983). Preferably, the activity of the enzyme is reduced to at most 50% of the wild-type level, more preferably no more than 25%, 10% or 5%, and most preferably is undetectable. The level of Yap3p proteolytic

activity may be measured by determining the production of the 45 kDa fragment, or by the ^{125}I - β_{h} -lipoprotein assay of Azaryan *et al* (1993), also used by Cawley *et al* (1993). Kex2p proteolytic activity may similarly be measured by known assays, for example as set out in Fuller *et al* (1989).

5

The albumin may be a human albumin, or a variant thereof, or albumin from any other animal.

By "variants" we include insertions, deletions and substitutions, either
10 conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin which include the region cleaved by Yap3p, for example those fragments disclosed in EP 322 094 (namely HSA (1-
15 n), where n is 369 to 419) which are sufficiently long to include the Yap3p-cleaved region (ie where n is 403 to 419); and fusions of albumin (or Yap3p-cleavable portions thereof) with other proteins, for example the kind disclosed in WO 90/13653.

20 By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and site-directed mutagenesis as described below.

25

A second aspect of the invention provides a modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with the *S. cerevisiae* yeast aspartyl protease 3 (Yap3p) when expressed in yeast, characterised in that the
30 modified albumin is not susceptible to such cleavage.

Preferably, the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein. Suitably, the said monobasic amino acid is arginine. Conveniently, the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin, especially any of Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg. Thus, in one particular embodiment, the naturally-occurring albumin is human albumin and the modified protein lacks Arg⁴¹⁰ and, optionally, one or both Lys⁴¹³Lys⁴¹⁴ lysines. For example, the modified albumin may be human albumin having the amino acid changes R410A, K413Q, K414Q. Equivalent modifications in bovine serum albumin include replacing the Arg⁴⁰⁸ and/or one or both of Arg⁴¹¹Lys⁴¹². The person skilled in the art will be able to identify monobasic sites and pairs of basic residues in other albumins without difficulty.

The numbering of the residues corresponds to the sequence of normal mature human albumin. If the albumin is a variant (for example a polymorphic form) having a net deletion or addition of residues N-terminal to the position identified, then the numbering refers to the residues of the variant albumin which are aligned with the numbered positions of normal albumin when the two sequences are so aligned as to maximise the apparent homology.

20

A third aspect of the invention provides a polynucleotide encoding such a modified albumin.

The DNA is expressed in a suitable yeast (either the DNA being for a modified albumin, or the yeast lacking the Yap3p function) to produce an albumin. Thus, the DNA encoding the albumin may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate yeast cell for the expression and production of the albumin.

30

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

5

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

10

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the albumin, which can then be recovered, as is
15 known.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating
20 plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps). Other yeast expression plasmids are disclosed in EP-A-258 067, EP-A-286 424 and EP-A-424 117.

25 The polynucleotide coding sequences encoding the modified albumin of the invention may have additional differences to those required to produce the modified albumin. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the
30 activity or immunogenicity of the albumin or which may improve its activity

or immunogenicity, as well as reducing its susceptibility to a Yap3p protease activity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle
5 (1985). Since such modified coding sequences can be obtained by the application of known techniques to the teachings contained herein, such modified coding sequences are within the scope of the claimed invention.

Exemplary genera of yeast contemplated to be useful in the practice of the
10 present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula* (now reclassified as *Pichia*), *Histoplasma*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospordium*, *Leucosporidium*, *Botryosascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of
15 *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* sp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* sp. are *K. fragilis* and *K. lactis*. Examples of *Hansenula* (*Pichia*) sp. are *H. polymorpha* (now *Pichia angusta*), *H. anomala* (now *P. anomala*) and *P. pastoris*. *Y. lipolytica* is an example of a suitable *Yarrowia*
20 species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. Suitable promoters for *S. cerevisiae* include those associated with
25 the *PGK1* gene, *GAL1* or *GAL10* genes, *CYCI*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and hybrid
30 promoters involving hybrids of parts of 5' regulatory regions with parts of 5'

regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are
5 the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990).

Methods of transforming *Pichia* for expression of foreign genes are taught in,
10 for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

15 The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being
20 *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be
25 those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADHI* gene is preferred.

The albumin is initially expressed with a secretion leader sequence, which may
30 be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae*

include that from the mating factor α polypeptide (MF α -1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. When the yeast strain lacks Kex2p activity (or equivalent) as well as being *yap3*, it may
5 be advantageous to choose a secretion leader which need not be cleaved from the albumin by Kex2p. Such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MF α -1, β -glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α -galactosidase (*MEL1*); *K. lactis*
10 killer toxin; and *Candida* glucoamylase.

Various non-limiting embodiments of the invention will now be described by way of example and with reference to the accompanying drawings in which:

15 Figure 1 is a general scheme for the construction of mutated rHA expression plasmids, in which HA is a human albumin coding sequence, L is a sequence encoding a secretion leader, P is the PRB1 promoter, T is the ADH1 terminator, amp is an ampicillin resistance gene and LEU2 is the leucine selectable marker;

20

Figure 2 is a drawing representing a Western blot analysis of mutant rHA secreted by *S. cerevisiae*, in which Track A represents the culture supernatant from DB1 cir^o pAYE316 (normal rHA), Track B represents the culture supernatant from DB1 cir⁺ pAYE464 (alteration 1), and Track C represents the
25 culture supernatant from DB1 cir⁺ pAYE468 (alteration 3);

Figure 3 is a scheme of the construction of pAYE515;

30 Figure 4 is a comparison of rHA fragment production by wild-type and protease-disrupted strains, presented as a drawing of an anti-HSA Western blot

of culture supernatant from shake flask cultures separated by non-reducing 10% SDS/PAGE, in which Track A corresponds to DB1 cir^o pAYE316, Track B corresponds to DXY10 cir^o pAYE316 (*yap3* strain), and Track C corresponds to ABB50 cir^o pAYE316 (*yap3*, *kex2* strain);

5

Figure 5 is similar to Figure 4 but shows Coomassie Brilliant Blue stained 12.5% SDS Phastgel (Pharmacia) of culture supernatants from fed batch fermentations, namely Track D for the HSA standard, Track E for DB1 cir^o pAYE316, Track F for DB1 Δ *kex2* cir^o pAYE522, and Track G for DXY10 cir^o pAYE522; and

10

Figure 6 is a scheme for the construction of pAYE519.

Detailed description of the invention

15

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding HSA are derived from the cDNA disclosed in EP 201 239.

20 **Example 1: Modification of the HSA cDNA.**

In order to investigate the role of endoproteases in the generation of rHA fragment, the HSA cDNA (SEQ1 (which includes a sequence encoding the artificial secretion leader sequence of WO 90/01063)) was modified by site-directed mutagenesis. Three separate changes were made to the HSA sequence

25 (SEQ2). The first, using the mutagenic primer FOG1, changed the Arg⁴¹⁰ codon only, replacing it with an Ala codon, leaving intact the dibasic site, Lys⁴¹³Lys⁴¹⁴. The second change, using primer FOG2, changed the residues 407-409, including the C-terminal residues of fragment, from LeuLeuVal to

30 AlaValAla. The third change, using the primer FOG3, altered residues 410-

414 from ArgTyrThrLysLys (SEQ3) to AlaTyrThrGlnGln (SEQ4). The oligonucleotides encoded not only the amino acid changes, but also conservative base changes that create either a *PvuII* or an *SpeI* restriction site in the mutants to facilitate detection of the changed sequences.

5

Single-stranded DNA of an M13mp19 clone, mp19.7 (EP 201 239; Figure 2), containing the HSA cDNA was used as the template for the mutagenesis reactions using the *In Vitro* Mutagenesis System, Version 2 (Amersham International plc) according to the manufacturer's instructions. Individual
10 plaques were selected and sequenced to confirm the presence of the mutations. Double stranded RF DNA was then made from clones with the expected changes and the DNA bearing the mutation was excised on an *XbaI/SacI* fragment (Figure 1). This was used to replace the corresponding wild-type
15 *XbaI/SacI* fragment within the plasmid was checked by digesting with *PvuII* or *SpeI* as appropriate. These *HindIII* fragments were excised and inserted into the expression vector pAYE219 (Figure 1) to generate the plasmids pAYE464 (alteration 1, R410A), pAYE470 (alteration 2, L407A, L408V, V409A) and pAYE468 (alteration 3, R410A, K413Q, K414Q). These expression plasmids
20 comprise the *S. cerevisiae PRB1* promoter (WO 91/02057) driving expression of the HSA/MF α 1 leader sequence (WO 90/01063) fused in-frame with the mutated HA coding sequence which is followed by the *ADHI* transcription terminator. The plasmids also contain part of the 2 μ m plasmid to provide replication functions and the *LEU2* gene for selection of transformants.

25

pAYE464, pAYE470 and pAYE468 were introduced into *S. cerevisiae* DB1 *cir*⁺ (*a*, *leu2*; Sleep *et al*, 1990) by transformation and individual transformants were grown for 3 days at 30°C in 10ml YEPS (1% w/v yeast extract, 2% w/v peptone, 2% w/v sucrose) and then the supernatants were examined by anti-HSA
30 Western blot for the presence of the rHA fragment. The Western blots clearly

showed that fragment was still produced by the strains harbouring pAYE464, although the level was reduced slightly compared to the control expressing wild-type rHA. The mutations in the plasmid pAYE470 appeared to have no effect on the generation of fragment. However, DB1 cir⁺ pAYE468 showed a novel pattern of HSA-related bands, with little or no fragment.

One example of each of DB1 cir⁺ pAYE464 and DB1 cir⁺ pAYE468 were grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 ± 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH ₂ PO ₄	114.0
MgSO ₄	12.0
CaCl ₂ .6H ₂ O	3.0
Na ₂ EDTA	2.0

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
ZnSO ₄ .7H ₂ O	3.0
FeSO ₄ .7H ₂ O	10.0
MnSO ₄ .4H ₂ O	3.2
CuSO ₄ .5H ₂ O	0.079
H ₃ BO ₃	1.5
KI	0.2
Na ₂ MoO ₄ .2H ₂ O	0.5
CoCl ₂ .6H ₂ O	0.56
H ₃ PO ₄	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, (NH₄)₂SO₄ 5 g/L, citric acid monohydrate 6.09 g/L, Na₂HPO₄ 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun,

Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h⁻¹). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved. The culture supernatants were compared with those of a wild-type rHA producer by Coomassie-stained SDS/PAGE and by Western blot. These indicated (Figure 2) that, whilst elimination of the monobasic Arg⁴¹⁰ (pAYE464) did reduce the level of the fragment by a useful amount, removal of both potential protease sites (pAYE468) almost abolished the 45kDa fragment.

The above data suggested that the generation of rHA fragment might be due to endoproteolytic attack, though the absence of an effect of removal of the potential Kex2p site Lys⁴¹³Lys⁴¹⁴ (Sleep *et al*, 1990, and confirmed by other studies not noted here) unless combined with elimination of Arg⁴¹⁰, had suggested a complex etiology. The reduction in the amount of fragment with the mutated rHA could in principle be due to an effect of the changes on the kinetics of folding of the molecule and not due to the removal of protease cleavage sites.

Example 2: Disruption of the YAP3 gene.

The YAP3 gene encoding yeast aspartyl protease 3 was mutated by the process of gene disruption (Rothstein 1983) which effectively deleted part of the YAP3 coding sequence, thereby preventing the production of active Yap3p.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the YAP3 gene (Egel-Mitani *et al*, 1990) were synthesised using an Applied

Biosystems 380B Oligonucleotide Synthesiser. To assist the reader, we include as SEQ15 the sequence of the *YAP3* gene, of which 541-2250 is the coding sequence.

5 **5' end**

YAP3A: 5'-CGTCAGACCTTGCATGCAGCCAAGACACCCTCACATAGC-3' (SEQ5)

YAP3B: 5'-CCGTTACGTTCTGTGGTGGCATGCCCACTTCCAAGTCCACCG-3' (SEQ6)

10

3' end

YAP3C: 5'-GCGTCTCATAGTGGAAAAGCTTCTAAATACGACAACTTCCCC-3' (SEQ7)

15 YAP3D: 5'-CCCAAATGGTACCTGTGTCATCACTCGTTGGGATAATACC-3' (SEQ8)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *YAP3* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc). Conditions were as follows: 2.5 µg/ml genomic DNA, 5 µg/ml of each primer, 20 denature at 94°C 61 seconds, anneal at 37°C 121 secs, extend at 72°C 181 secs for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size. The 5' fragment 25 was digested with *SphI* and cloned into the *SphI* site of pUC19HX (pUC19 lacking a *HindIII* site) to give pAYE511 (Figure 3), in which the orientation is such that *YAP3* would be transcribed towards the *KpnI* site of the pUC19HX polylinker. The 3' *YAP3* fragment was digested with *HindIII* and *Asp718* (an isoschizomer of *KpnI*) and ligated into pUC19 digested with *HindIII/Asp718* to 30 give pAYE512. Plasmid DNA sequencing was carried out on the inserts to confirm that the desired sequences had been cloned. The *HindIII/Asp718* fragment of pAYE512 was then subcloned into the corresponding sites of pAYE511 to give pAYE513 (Fig 3), in which the 5' and 3' regions of *YAP3*

are correctly orientated with a unique *Hind*III site between them. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hind*III fragment and then inserted into this site to give pAYE515 (Fig 3), with *URA3* flanked by the 5' and 3' regions of *YAP3*, and transcribed in the opposite direction to *YAP3*.

5

A *ura3* derivative of strain DB1 *cir*^o pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). The strain was grown overnight in 100 mL buffered minimal medium and the cells were collected by centrifugation and then washed once with sterile water. The cells were then resuspended in 10 mL sterile water and 2 mL aliquots were placed in separate 15 mL Falcon tubes. A 5 mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0 μ L, 20 μ L, 40 μ L, 80 μ L or 160 μ L. The cells were then incubated at 30°C for 30 min and then centrifuged and washed three times with sterile water. Finally, the cells were resuspended in 1 mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were grown on YEP plates containing 2% w/v sucrose and then replica-plated onto YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1 mg/mL) and uracil (50 μ g/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by introduction of the *URA3* gene by transformation. One such strain, DBU3 *cir*^o pAYE316, was transformed with the *Sph*I/*Asp*718 *YAP3-URA3-YAP3* fragment of pAYE515 with selection for Ura⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *YAP3* gene and confirmed the disruption of the *YAP3* gene. An anti-HSA Western blot of

30

YEPS shake-flask supernatants of two transformants indicated that disruption of *YAP3* markedly reduced rHA fragment levels.

5 One *yap3* derivative of DBU3 cir° pAYE316, designated DXY10 cir° pAYE316, was grown several times by fed-batch fermentation in minimal medium to high cell dry weight. When supernatants were examined by Coomassie-stained PAGE and anti-HSA Western blot (Figs 4 and 5), the reduction in the level of rHA 45kDa fragment was clearly apparent; estimates of the amount of the degradation product vary from $1/3$ to $1/5$ of the levels seen
10 with the *YAP3* parent. The amount of rHA produced was not adversely affected by the *yap3* mutation, indeed DXY10 cir° pAYE316 was found to produce 30-50% more rHA than the *YAP3* equivalent, DB1 cir° pAYE316. Despite the fact that cleavage of the leader sequence from the HA sequence is C-terminal to a pair of basic residues, the rHA was found to have the correct
15 N-terminus.

The fermentation broth was centrifuged to remove the cells and then subject to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which
20 was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0, at which point it was >95% pure. It may be purified further by techniques known in the art.

25 The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

Example 3: Disruption of the *KEX2* gene in a *yap3* strain.

To construct a strain lacking both Yap3p and Kex2p activity, a *lys2* derivative of yeast strain DXY10 cir^o (pAYE316) was obtained by random chemical
 5 mutagenesis and selection for resistance to α -amino adipate (Barnes and Thorner, 1985). Cells were mutagenised as in Example 2 and then plated on YNB minimal medium containing 2% w/v sucrose and supplemented with 2 mg/mL DL- α -amino adipate as the sole nitrogen source and 30 μ g/mL lysine. Colonies able to grow on this medium were purified and tested to verify that
 10 they were unable to grow in the absence of lysine supplementation and that this defect could be corrected by the introduction of the *LYS2* gene by transformation. This strain was then mutated by the process of gene disruption which effectively disrupted part of the *KEX2* coding sequence, thereby preventing production of active Kex2p. To assist the reader, the sequence of
 15 the *KEX2* gene is reproduced herein as SEQ14, of which 1329-3773 is the coding sequence.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the *KEX2* gene (Fuller *et al*, 1989) were synthesised using an Applied
 20 Biosystems 380B Oligonucleotide Synthesiser.

5' end

KEX2A: 5'-CCATCTGGATCCAATGGTGCTTTGGCCAAATAAATAGTTTCAGC-3'
 (SEQ9)
 25 KEX2B: 5'-GCTTCTTTTACCGGTAACAAGCTTGAGTCCATTGG-3'
 (SEQ10)

3' end

KEX2C: 5'-GGTAAGGTTTAGTCGACCTATTTTTGTTTTGTCTGC-3'
 (SEQ11)
 30 KEX2D: 5'-GGAAACGTATGAATTCGATATCATTGATACAGACTCTGAGTACG-3'
 (SEQ12)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *KEX2* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories Inc). Conditions were as follows: 2.5 µg/ml genomic DNA, 5 µg/ml of each primer, denature 94°C 61s, anneal 37°C 121s, extend 72°C 181s for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size (0.9 kb for the 5' product and 0.62 kb for the 3' product). The 5' product was digested with *Bam*HI and *Hind*III and the 3' product was digested with *Hind*III and *Sal*I and then the two fragments were together cloned into pUC19HX digested with *Bam*HI and *Sal*I. A 4.8 kb *Hind*III fragment comprising the *S. cerevisiae* *LYS2* gene (Barnes & Thorner, 1985) was then inserted into the resulting plasmid at *Hind*III (ie between the two *KEX2* fragments) to form pAYE519 (Fig 6).

15

The *lys2* derivative of DXY10 cir^o (pAYE316), *lys2-16*, was transformed with the 6.0 kb *KEX2-LYS2-KEX2* fragment of pAYE519, selecting for Lys⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *KEX2* gene and confirmed the disruption of the *KEX2* gene. An anti-HSA Western blot of YEPS shake-flask culture supernatants of these transformants indicated that disruption of *KEX2* in a *yap3* strain reduced the level of rHA fragment still further, despite the lack of an effect of disruption of *KEX2* alone in Example 4 below. Analysis of the rHA produced by one such strain, ABB50, indicated that the leader sequence was incorrectly processed, leading to an abnormal N-terminus.

The strain ABB50 (pAYE316) was cured of its plasmid (Sleep *et al*, 1991) and transformed with a similar plasmid, pAYE522, in which the hybrid leader sequence was replaced by the *S. cerevisiae* invertase (*SUC2*) leader sequence

30

The method of Southern hybridization used for detection of the *YAP3* homologue can be adapted to clone the gene sequence from a genomic DNA library of *Pichia* DNA using standard procedures (Sambrook *et al*, 1989). Disruption of the *YAP3* homologue in *Pichia sp.* can be achieved using similar
5 techniques to those used above for *Saccharomyces* (Cregg and Madden, 1987).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Delta Biotechnology Limited
- (B) STREET: Castle Court, Castle Boulevard
- (C) CITY: Nottingham
- (D) STATE: Nottinghamshire
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast strains and modified albumins

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 73..1827

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATGAAGTGGG TAAGCTTTAT TTCCCTTCTT TTTCTCTTTA GCTCGGCTTA TTCCAGGAGC      60
TTGGATAAAA GA GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA      108
           Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys
             1             5             10

GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT GCC TTT GCT      156
Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala
           15             20             25

CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT      204
Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn
           30             35             40

GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA      252
Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu
           45             50             55             60

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AAT	TGT	GAC	AAA	TCA	CTT	CAT	ACC	CTT	TTT	GGA	GAC	AAA	TTA	TGC	ACA	300
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			65						70					75		
GTT	GCA	ACT	CTT	CGT	GAA	ACC	TAT	GGT	GAA	ATG	GCT	GAC	TGC	TGT	GCA	348
Val	Ala	Thr	Leu	Arg	Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	
			80					85					90			
AAA	CAA	GAA	CCT	GAG	AGA	AAT	GAA	TGC	TTC	TTG	CAA	CAC	AAA	GAT	GAC	396
Lys	Gln	Glu	Pro	Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	
		95					100					105				
AAC	CCA	AAC	CTC	CCC	CGA	TTG	GTG	AGA	CCA	GAG	GTT	GAT	GTG	ATG	TGC	444
Asn	Pro	Asn	Leu	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	
	110					115					120					
ACT	GCT	TTT	CAT	GAC	AAT	GAA	GAG	ACA	TTT	TTG	AAA	AAA	TAC	TTA	TAT	492
Thr	Ala	Phe	His	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	
	125				130					135					140	
GAA	ATT	GCC	AGA	AGA	CAT	CCT	TAC	TTT	TAT	GCC	CCG	GAA	CTC	CTT	TTC	540
Glu	Ile	Ala	Arg	Arg	His	Pro	Tyr	Phe	Tyr	Ala	Pro	Glu	Leu	Leu	Phe	
				145					150					155		
TTT	GCT	AAA	AGG	TAT	AAA	GCT	GCT	TTT	ACA	GAA	TGT	TGC	CAA	GCT	GCT	588
Phe	Ala	Lys	Arg	Tyr	Lys	Ala	Ala	Phe	Thr	Glu	Cys	Cys	Gln	Ala	Ala	
			160					165					170			
GAT	AAA	GCT	GCC	TGC	CTG	TTG	CCA	AAG	CTC	GAT	GAA	CTT	CGG	GAT	GAA	636
Asp	Lys	Ala	Ala	Cys	Leu	Leu	Pro	Lys	Leu	Asp	Glu	Leu	Arg	Asp	Glu	
		175					180					185				
GGG	AAG	GCT	TCG	TCT	GCC	AAA	CAG	AGA	CTC	AAG	TGT	GCC	AGT	CTC	CAA	684
Gly	Lys	Ala	Ser	Ser	Ala	Lys	Gln	Arg	Leu	Lys	Cys	Ala	Ser	Leu	Gln	
	190					195					200					
AAA	TTT	GGA	GAA	AGA	GCT	TTC	AAA	GCA	TGG	GCA	GTA	GCT	CGC	CTG	AGC	732
Lys	Phe	Gly	Glu	Arg	Ala	Phe	Lys	Ala	Trp	Ala	Val	Ala	Arg	Leu	Ser	
	205				210				215					220		
CAG	AGA	TTT	CCC	AAA	GCT	GAG	TTT	GCA	GAA	GTT	TCC	AAG	TTA	GTG	ACA	780
Gln	Arg	Phe	Pro	Lys	Ala	Glu	Phe	Ala	Glu	Val	Ser	Lys	Leu	Val	Thr	
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GAT	CTT	ACC	AAA	GTC	CAC	ACG	GAA	TGC	TGC	CAT	GGA	GAT	CTG	CTT	GAA	828
Asp	Leu	Thr	Lys	Val	His	Thr	Glu	Cys	Cys	His	Gly	Asp	Leu	Leu	Glu	
			240					245					250			
TGT	GCT	GAT	GAC	AGG	GCG	GAC	CTT	GCC	AAG	TAT	ATC	TGT	GAA	AAT	CAA	876
Cys	Ala	Asp	Asp	Arg	Ala	Asp	Leu	Ala	Lys	Tyr	Ile	Cys	Glu	Asn	Gln	
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GAT	TCG	ATC	TCC	AGT	AAA	CTG	AAG	GAA	TGC	TGT	GAA	AAA	CCT	CTG	TTG	924
Asp	Ser	Ile	Ser	Ser	Lys	Leu	Lys	Glu	Cys	Cys	Glu	Lys	Pro	Leu	Leu	
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GAA	AAA	TCC	CAC	TGC	ATT	GCC	GAA	GTG	GAA	AAT	GAT	GAG	ATG	CCT	GCT	972
Glu	Lys	Ser	His	Cys	Ile	Ala	Glu	Val	Glu	Asn	Asp	Glu	Met	Pro	Ala	
	285				290					295				300		
GAC	TTG	CCT	TCA	TTA	GCT	GCT	GAT	TTT	GTT	GAA	AGT	AAG	GAT	GTT	TGC	1020
Asp	Leu	Pro	Ser	Leu	Ala	Ala	Asp	Phe	Val	Glu	Ser	Lys	Asp	Val	Cys	
			305						310					315		
AAA	AAC	TAT	GCT	GAG	GCA	AAG	GAT	GTC	TTC	CTG	GCC	ATG	TTT	TTG	TAT	1068
Lys	Asn	Tyr	Ala	Glu	Ala	Lys	Asp	Val	Phe	Leu	Gly	Met	Phe	Leu	Tyr	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 585 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
 1 5 10 15
 Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
 20 25 30
 Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
 35 40 45
 Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
 50 55 60
 Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
 65 70 75 80
 Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
 85 90 95
 Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
 100 105 110
 Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
 115 120 125
 Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
 130 135 140
 Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
 145 150 155 160
 Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
 165 170 175
 Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
 180 185 190
 Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
 195 200 205
 Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
 210 215 220
 Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
 225 230 235 240
 Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
 245 250 255
 Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
 260 265 270
 Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
 275 280 285
 Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser

290		295		300
Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala				
305		310		320
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg				
		325		335
Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr				
		340		350
Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu				
		355		365
Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro				
		370		380
Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu				
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Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro				
		405		415
Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys				
		420		430
Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys				
		435		445
Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His				
		450		460
Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser				
		465		480
Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr				
		485		495
Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp				
		500		510
Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala				
		515		525
Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu				
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Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys				
		545		560
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val				
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Ala Ala Ser Gln Ala Ala Leu Gly Leu				
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(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Tyr Thr Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Tyr Thr Gln Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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39

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTTACGTT CTGTGGTGGC ATGCCCACTT CCAAGTCCAC CG

42

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 42 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTTCATA GTGGAAAAGC TTCTAAATAC GACAACTTCC CC

42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCAAATGG TACCTGTGTC ATCACTCGTT GGGATAATAC C

41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTGGAT CCAATGGTGC TTTGGCCAAA TAAATAGTTT CAGC

44

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTTCTTTTA CCGGTAACAA GCTTGAGTCC ATTGG

35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 37 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTAAGGTTT AGTCGACCTA TTTTTTGTTT TGTCTGC

37

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGAAACGTAT GAATTCGATA TCATTGATAC AGACTCTGAG TACG

44

(2) INFORMATION FOR SEQ ID NO: 13:

ACTCCGGTAA	AGACGGATTA	TACGTGATGA	CTCAAAGTA	CTTCGAGGAG	TACTGCTTTC	720
AAATTGTGGT	CGATATCAAT	GAATTGCCAA	AAGAGCTGGC	TTCAAATTC	ACCTCAGGTA	780
AGGAAGAGCC	GATTGTCTTG	CCCATCTGGA	CCCAATGGTG	CTTTGGCCAA	ATAAATAGTT	840
TCAGCAGCTC	TGATGTAGAT	ACACGTATCT	CGACATGTTT	TATTTTACT	ATACATACAT	900
AAAAGAAATA	AAAAATGATA	ACGTGTATAT	TATTATTCAT	ATAATCAATG	AGGGTCATTT	960
TCTGAAACGC	AAAAAACGGT	AAATGGAAAA	AAAATAAAGA	TAGAAAAAGA	AAACAAACAA	1020
AGGAAAAGTT	AGCATATTAA	ATAACTGAGC	TGATACTTCA	ACAGCATCGC	TGAAGAGAAC	1080
AGTATTGAAA	CCGAAACATT	TTCTAAAGGC	AAACAAGGTA	CTCCATATTT	GCTGGACGTG	1140
TTCTTTCTCT	CGTTTCATAT	GCATAATTCT	GTCATAAGCC	TGTTCTTTTT	CCTGGCTTAA	1200
ACATCCCGTT	TTGTAAGA	GAAATCTATT	CCACATATTT	CATTCATTCG	GCTACCATAC	1260
TAAGGATAAA	CTAATCCCGT	TGTTTTTTGG	CCTCGTCACA	TAATTATAAA	CTACTAACCC	1320
ATTATCAGAT	GAAAGTGAGG	AAATATATTA	CTTTATGCTT	TTGGTGGGCC	TTTCAACAT	1380
CCGCTCTTGT	ATCATCACAA	CAAATCCCAT	TGAAGGACCA	TACGTCACGA	CAGTATTTTG	1440
CTGTAGAAAAG	CAATGAAACA	TTATCCCGCT	TGGAGGAAAT	GCATCCAAAT	TGGAAATATG	1500
AACATGATGT	TCGAGGGCTA	CCAAACCATT	ATGTTTTTTC	AAAAGAGTTG	CTAAAATTGG	1560
GCAAAGATC	ATCATTAGAA	GAGTTACAGG	GGGATAACAA	CGACCACATA	TTATCTGTCC	1620
ATGATTTATT	CCCGCGTAAC	GACCTATTTA	AGAGACTACC	GGTGCCTGCT	CCACCAATGG	1680
ACTCAAGCTT	GTTACCGGTA	AAAGAAGCTG	AGGATAAACT	CAGCATAAAT	GATCCGCTTT	1740
TTGAGAGGCA	GTGGCACTTG	GTCAATCCAA	GTTTTCTGG	CAGTGATATA	AATGTTCTTG	1800
ATCTGTGGTA	CAATAATATT	ACAGGCGCAG	GGGTCGTGGC	TGCCATTGTT	GATGATGGCC	1860
TTGACTACGA	AAATGAAGAC	TTGAAGGATA	ATTTTTGCGC	TGAAGGTCT	TGGGATTTC	1920
ACGACAATAC	CAATTTACCT	AAACCAAGAT	TATCTGATGA	CTACCATGGT	ACGAGATGTG	1980
CAGGTGAAAT	AGCTGCCAAA	AAAGGTAACA	ATTTTTGCGG	TGTCGGGGTA	GGTTACAACG	2040
CTAAAATCTC	AGGCATAAGA	ATCTTATCCG	GTGATATCAC	TACGGAAGAT	GAAGCTGCGT	2100
CCTTGATTTA	TGGTCTAGAC	GTAACGATA	TATATTCATG	CTCATGGGGT	CCCGCTGATG	2160
ACGGAAGACA	TTTACAAGGC	CCTAGTGACC	TGGTGAAAAA	GGCTTTAGTA	AAAGGTGTTA	2220
CTGAGGGAAG	AGATTCCAAA	GGAGCGATTT	ACGTTTTTGC	CAGTGGAAT	GGTGGAATC	2280
GTGGTGATAA	TTGCAATTAC	GACGGCTATA	CTAATTCAT	ATATTCTATT	ACTATTGGGG	2340
CTATTGATCA	CAAAGATCTA	CATCCTCCTT	ATTCCGAAGG	TTGTTCCGCC	GTCATGGCAG	2400
TCACGTATTC	TTCAGGTTCA	GGCGAATATA	TTCATTCGAG	TGATATCAAC	GGCAGATGCA	2460
GTAATAGCCA	CGGTGGAACG	TCTGCGGCTG	CTCCATTAGC	TGCCGGTGT	TACACTTGT	2520
TACTAGAAGC	CAACCCAAAC	CTAACTGGA	GAGACGTACA	GTATTTATCA	ATCTGTCTG	2580
CGGTAGGGTT	AGAAAAGAAC	GCTGACGGAG	ATTGGAGAGA	TAGCGCCATG	GGGAAGAAAT	2640

ACTCTCATCG CTATGGCTTT GGTAAAATCG ATGCCCATAA GTTAATTGAA ATGTCCAAGA	2700
CCTGGGAGAA TGTTAACGCA CAAACCTGGT TTTACCTGCC AACATTGTAT GTTCCCAGT	2760
CCACAAACTC CACGGAAGAG ACATTAGAAT CCGTCATAAC CATATCAGAA AAAAGTCTTC	2820
AAGATGCTAA CTTCAAGAGA ATTGAGCACG TCACGGTAAC TGTAGATATT GATACAGAAA	2880
TTAGGGGAAC TACGACTGTC GATTTAATAT CACCAGCGGG GATAATTCA AACCTGGCG	2940
TTGTAAGACC AAGAGATGTT TCATCAGAGG GATTCAAAGA CTGGACATTC ATGTCTGTAG	3000
CACATTGGGG TGAGAACGGC GTAGGTGATT GGAAAATCAA GGTTAAGACA ACAGAAAATG	3060
GACACAGGAT TGACTTCCAC AGTTGGAGGC TGAAGCTCTT TGGGGAATCC ATTGATTCAT	3120
CTAAAACAGA AACTTTCGTC TTTGGAAACG ATAAAGAGGA GGTGAACCA GCTGCTACAG	3180
AAAGTACCGT ATCACAATAT TCTGCCAGTT CAACTTCTAT TTCCATCAGC GCTACTTCTA	3240
CATCTTCTAT CTCAATTGGT GTGGAAACGT CGGCCATTCC CCAAACGACT ACTGCGAGTA	3300
CCGATCCTGA TTCTGATCCA AACACTCCTA AAAAATTTC CTCTCCTAGG CAAGCCATGC	3360
ATTATTTTTT AACAAATATT TTGATTGGCG CCACATTTTT GGTGTTATAC TTCATGTTTT	3420
TTATGAAATC AAGGAGAAGG ATCAGAAGGT CAAGAGCGGA AACGTATGAA TTCGATATCA	3480
TTGATACAGA CTCTGAGTAC GATTCTACTT TGGACAATGG AACTCCGGA ATTACTGAGC	3540
CCGAAGAGGT TGAGGACTTC GATTTTGATT TGTCCGATGA AGACCATCTT GCAAGTTTGT	3600
CTTCATCAGA AAACGGTGAT GCTGAACATA CAATTGATAG TGTACTAACA AACGAAAATC	3660
CATTTAGTGA CCCTATAAAG CAAAAGTTCC CAAATGACGC CAACGCAGAA TCTGCTTCCA	3720
ATAAATTACA AGAATTACAG CCTGATGTTT CTCCATCTTC CGGACGATCG TGATTGATA	3780
TGTACAGAAA GCTTCAAATT ACAAATAGC ATTTTTTCT TATAGATTAT AATACTCTCT	3840
CATACGTATA CGTATATGTG TATATGATAT ATAAACAAAC ATTAATATCC TATTCCTTCC	3900
GTTTGAAATC CCTATGATGT ACTTTGCATT GTTGCACCC GCGAATAAAA TGAAAATCC	3960
GAACCGATAT ATCAAGCACA TAAAAGGGGA GGTCCAATT AATGCATATT TAAGACCACA	4020
GCTGAATAAC TTTAAACGG CAGACAAAAC AAAAAATAGG TCGAATAAAC CTTACCTGCC	4080
TAGAAGGAAT GACAGCAGCT AATAAG	4106

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2526 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCGTTTTCTT TTCGTAAAA AAAACAATAG ACACTATATA TAGACACTTT TTCCTTTCCT	60
TCTTTGCGCG ATTTCAAGAG GAAAAGCATA CTAAATAAG AATATTCCTA AAACACACGT	120
TCTGACGCGT CAATTAGATC GTCAGACCTT GCATGCAGCC AAGACACCCT CACATAGCAC	180
TGCCTCCTTC CTCCTCTTTT CTGTCACCAC CTCACCTCCC TCGTCCACTC AACTGAGTGG	240
CTTTTCGCTC CTTTTATACT GCGCCATGAG TAGTTTTCGT TTCACTGATG TGTCCGAAAA	300
AATTGAGGTT TCATAAAAAA ATTCGTGGAC TTATTTATGG AGAAACAGGG AAATCCGACT	360
ACTTAAGAAA AGGGTGTCAA AGAGGATTTA CTTTTTTCCT TCTTTTTCGA TTTGTTCCCTA	420
TTCCGCAAT TGGACGGTTA TTAAGAAGAA CGCAATTGGC TTTTCTGTAT ATTAATAATAC	480
ATAGCGTAAT AAAAAGATAA GGTGAACACC AAGCATATAG TATAATATTA CCTACCACAT	540
ATGAAACTGA AAACGTAAAG ATCTGCGGTC CTTTCGTAC TCTTTGCATC GCAGGTTCTC	600
GGTAAGATAA TACCAGCAGC AAACAAGCGC GACGACGACT CGAATTCCAA GTTCGTCAAG	660
TGCCCCTTC ATAAGCTTTA CGGGGACTCG CTAGAAAATG TGGGAAGCGA CAAAAACCG	720
GAAGTACGCC TATTGAAGAG GGCTGACGGT TATGAAGAAA TTATAATTAC CAACCAGCAA	780
AGTTTCTATT CGGTGGACTT GGAAGTGGGC ACGCCACCAC AGAACGTAAC GGTCTGGTG	840
GACACAGGCT CCTCTGATCT ATGGATTATG GGCTCGGATA ATCCATACTG TTCTTCGAAC	900
AGTATGGGTA GTAGCCGGAG ACGTGTTATT GACAAACGTG ATGATTCGTC AAGCGGCGGA	960
TCTTTGATTA ATGATATAAA CCCATTTGGC TGTTGACGG GAACGGGCAG TGCCATTGGC	1020
CCCCTGCTA CGGGCTTAGG AGGCGGTTCA GGTACGGCAA CTCAATCCGT GCCTGCTTCG	1080
GAAGCCACCA TGGACTGTCA ACAATACGGG ACATTTTCCA CTTCCGGGCTC TTCTACATTT	1140
AGATCAAACA ACACCTATTT CAGTATTAGC TACGGTGATG GGACTTTTGC CTCCGGTACT	1200
TTTGGTACGG ATGTTTTGGA TTTAAGCGAC TTGAACGTTA CCGGGTTGTC TTTTGCCGTT	1260
GCCAAATGAAA CGAATTCTAC TATGGGTGTG TTAGGTATTG GTTTGCCCGA ATTAGAAGTC	1320
ACTTATTCTG GCTCTACTGC GTCTCATAGT GGAAAAGCTT ATAAATACGA CAACTTCCCC	1380
ATTGTATTGA AAAATTCTGG TGCTATCAA AGCAACACAT ATTCTTTGTA TTTGAACGAC	1440
TCGGACGCTA TGCATGGCAC CTTTTGTTC GGAGCCGTGG ACCACAGTAA ATATACGGGC	1500
ACCTTATACA CAATCCCCAT CGTAAACACT CTGAGTGCTA GTGGATTTAG CTCTCCCATT	1560
CAATTTGATG TCACTATTAA TGGTATCGGT ATTAGTGATT CTGGGAGTAG TAACAAGACC	1620
TTGACTACCA CTAAATAACC TGCTTTGTCT GATTCCGGTA CTACTTTGAC TTATTTACCT	1680
CAAACAGTGG TAAGTATGAT CGCTACTGAA CTAGGTGCGC AATACTCTTC CAGGATAGGG	1740
TATTACGTAT TGGACTGTCC ATCTGATGAT AGTATGGAAA TAGTGTTCCA TTTTGGTGGT	1800
TTTCACATCA ATGCACCACT TTCGAGTTTT ATCTTGAGTA CTGGCACTAC ATGTCTTTTA	1860
GGTATTATCC CAACGAGTGA TGACACAGGT ACCATTTTGG GTGATTCATT TTTGACTAAC	1920

GCGTACGTGG	TTTATGATTT	GGAGAATCTT	GAAATATCCA	TGGCACAAGC	TCGCTATAAT	1980
ACCACAAGCG	AAAATATCGA	AATTATCACA	TCCTCTGTTC	CAAGCGCCGT	AAAGGCACCA	2040
GGCTATACAA	ACACTTGGTC	CACAAGTGCA	TCTATTGTTA	CCGGTGGTAA	CATATTTACT	2100
GTAATTCCT	CACAAACTGC	TTCCTTTAGC	GGTAACCTGA	CGACCAGTAC	TGCATCCGCC	2160
ACTTCTACAT	CAAGTAAAAG	AAATGTTGGT	GATCATATAG	TTCCATCTTT	ACCCCTCACA	2220
TTAATTTCTC	TTCTTTTTGC	ATTCATCTGA	AAACCGTGC	ACAAAGTTTA	GACATTCACA	2280
TCTCCAAGCC	AGTTGGAGTT	TCTGGCGGAA	ATCGTTGCTC	TCGCTTGGGC	AAAGTTTTTT	2340
TTTATTATTA	ATTTTTTATT	GTTACGTTGG	CGGTCTTTAT	TTTACTTCA	CAATAGTTTA	2400
TCTTACCCAC	TAAGAATAGG	TTACCATTTA	TTCACATTTT	TTTTTCTCAT	TCCTAGTATA	2460
CTATTTACCT	GGGATATGGC	CTATAATCAA	AGGCTTTAAT	ATTCTAATAA	TTCGTTTGGC	2520
ATCTAG						2526

CLAIMS

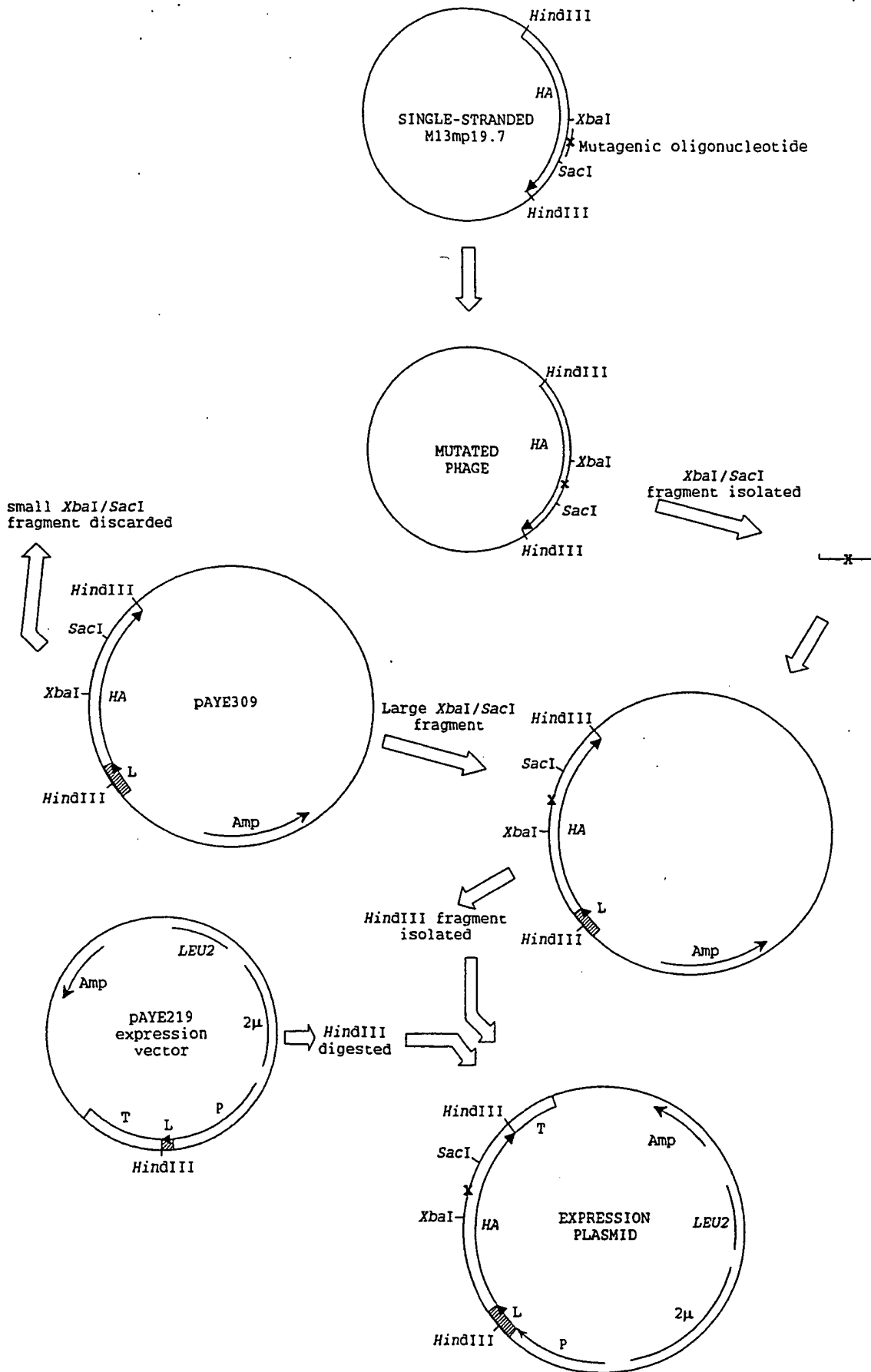
1. A process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.
2. A process according to Claim 1 wherein the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.
3. A process according to Claim 1 or 2 wherein the yeast is *S. cerevisiae*.
4. A process according to Claim 1, 2 or 3 wherein the yeast lacks a functional *YAP3* gene or homologue thereof.
5. A process according to any one of Claims 1 to 4 wherein the yeast cells additionally have a reduced level of *S. cerevisiae* Kex2p proteolytic activity.
6. A process according to any one of the preceding claims wherein the albumin is a human albumin.
7. A culture of yeast cells containing a polynucleotide sequence encoding an albumin and a second polynucleotide sequence encoding a secretion signal causing albumin expressed from the first polynucleotide sequence to be secreted from the yeast, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic

activity.

8. A culture according to Claim 7 wherein the albumin is a human albumin.
- 5
9. A culture according to Claim 7 or 8 wherein the yeast is *S. cerevisiae*.
10. A culture according to any one of Claims 7 to 9 wherein the said
10 signal is cleaved by the yeast prior to release of the albumin from the yeast.
11. A culture according to any one of Claims 7 to 10 wherein the yeast cells additionally have a reduced level of Kex2p proteolytic activity.
- 15
12. A culture according to Claim 11 wherein the said secretion signal is cleaved from the albumin by a protease other than Kex2p.
13. A modified albumin having at least 90% sequence identity to a
20 naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with yeast aspartyl protease 3 (Yap3p) when expressed and secreted in yeast, characterised in that the modified albumin is not susceptible to such cleavage.
- 25 14. A modified albumin according to Claim 13 wherein the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein.
15. A modified albumin according to Claim 13 or 14 wherein the said
30 monobasic amino acid is arginine.

16. A modified albumin according to Claim 14 or 15 wherein the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin.
- 5 17. A modified albumin according to Claim 16 wherein the said pair of amino acids is Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg.
18. A modified albumin according to Claim 13 wherein the naturally-occurring albumin is a human albumin and the modified protein lacks
10 Arg⁴¹⁰; and, optionally, residues 413 and 414 do not each consist of lysine or arginine.
19. A modified albumin according to Claim 18 which is a human albumin having the amino acid changes R410A, K413Q, K414Q.
15
20. A polynucleotide encoding a modified albumin according to any one of Claims 13 to 19.
21. A yeast containing a polynucleotide according to Claim 20,
20 transcription signals such that the modified albumin is expressed in the yeast, and a further polynucleotide adjacent the said polynucleotide such that the modified albumin is secreted from the yeast.

1/5
Figure 1.



2/5
Figure 2

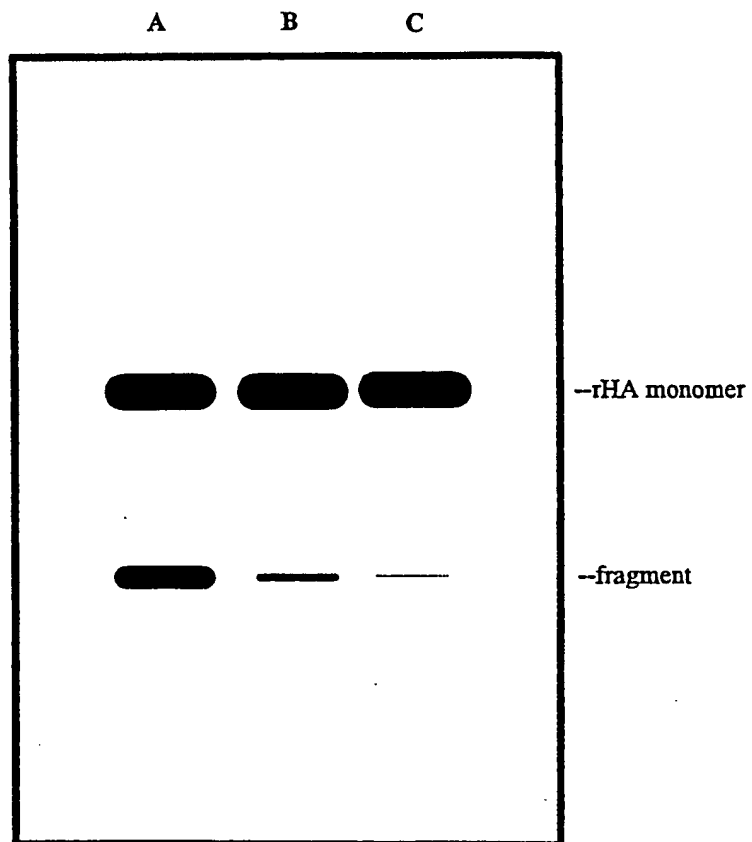


Figure 3

5' and 3' regions of YAP3 obtained by PCR:

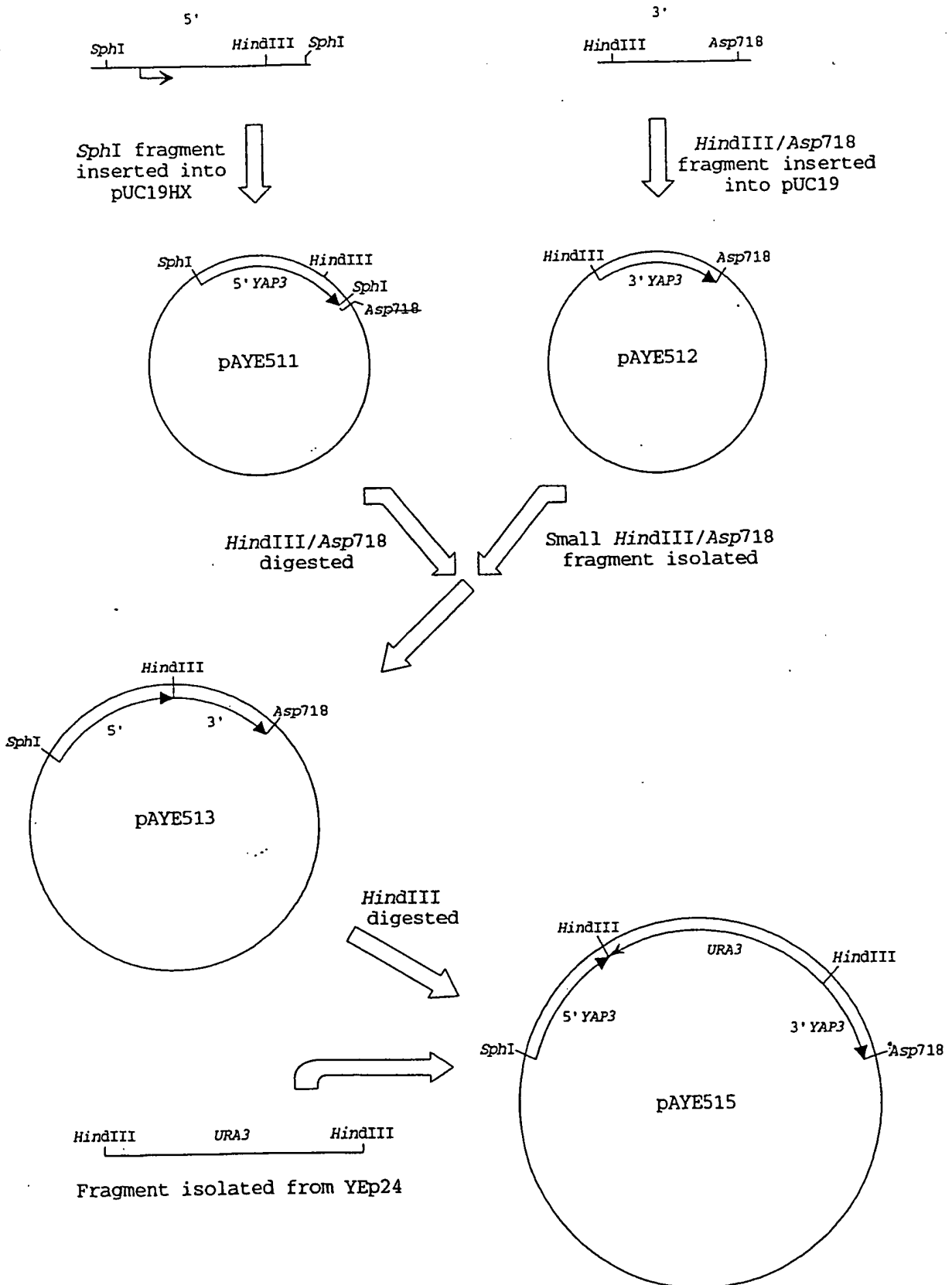


Figure 4

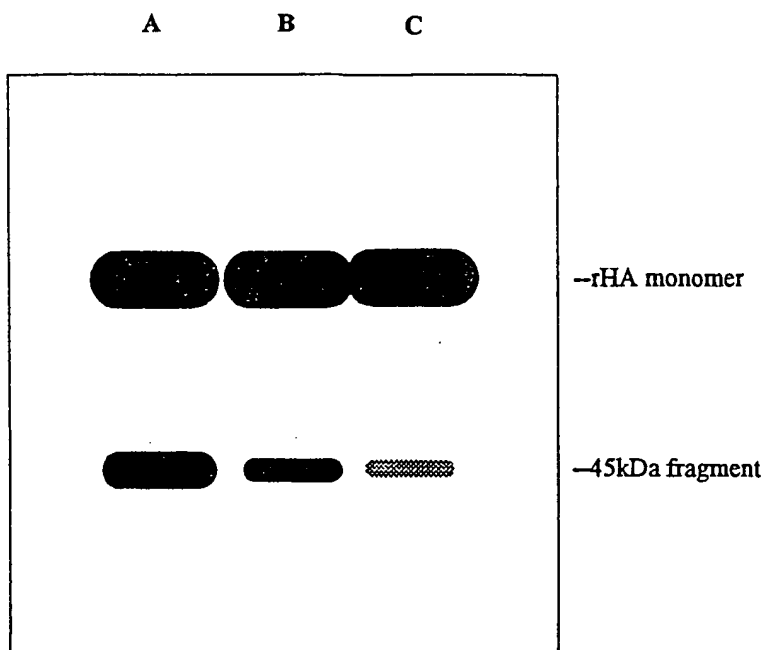
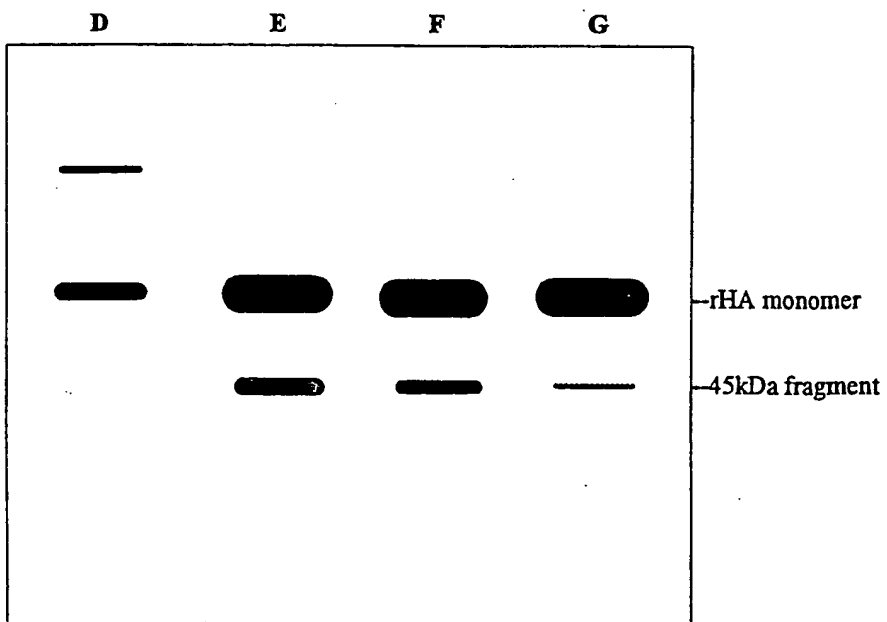
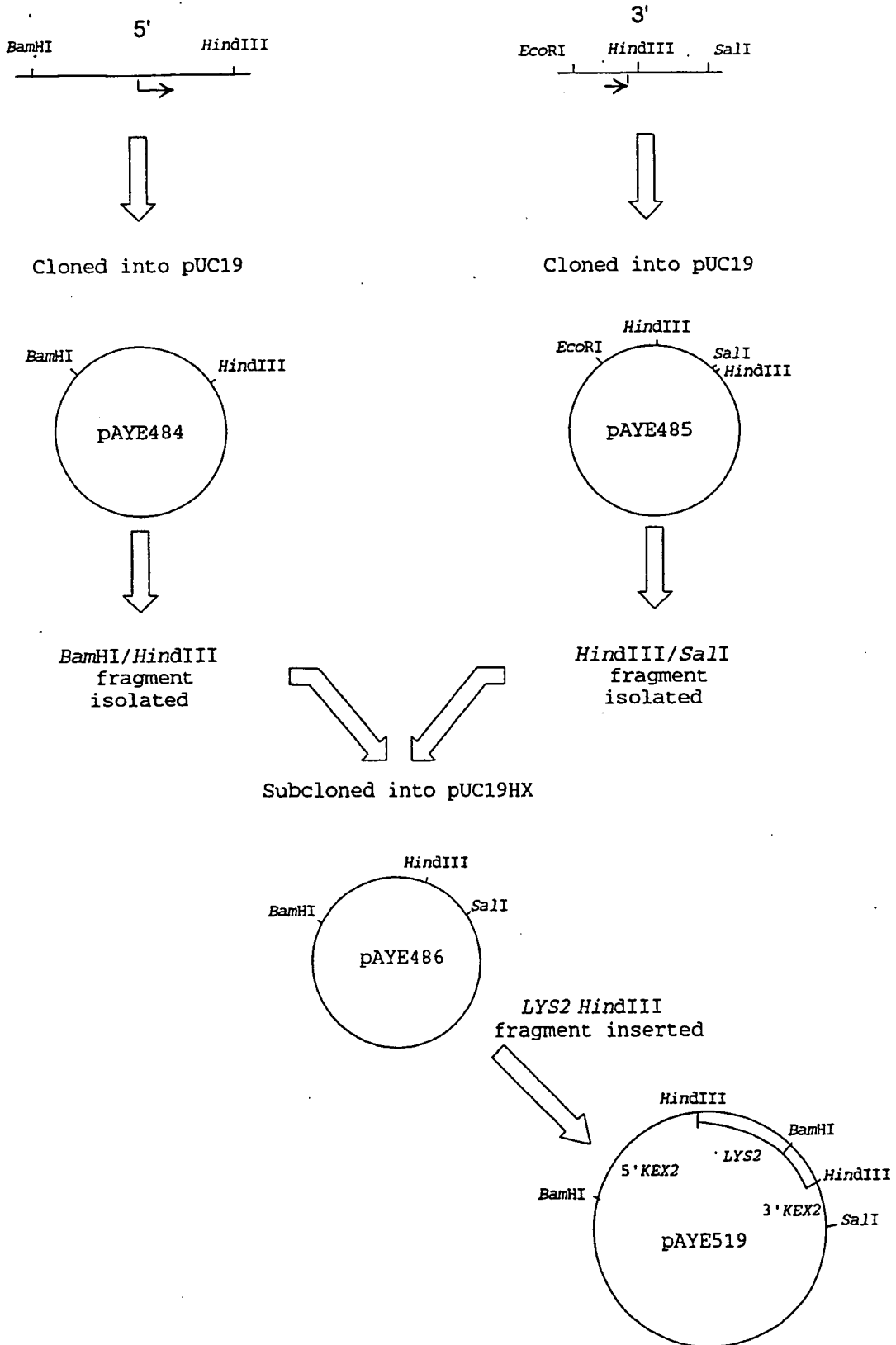


Figure 5



5/5
Figure 6

5' and 3' regions of KEX2 obtained by PCR:



A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/12 C12N1/21 A61K38/38 C12P21/02 C12N15/11 C12N1/20		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A61K C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	BIOCHIMIE, vol. 76, 1994 pages 226-233, Y. BOURBONNAIS ET AL. 'Cleavage of prosomatostatins by the yeast Yap3 and Kex2 endoprotease' *see the whole article* ---	1-21
Y	BIOTECHNOLOGY, vol. 8, no. 1, 1990 pages 42-46, D. SLEEP ET AL. 'The secretion of human serum albumin from the yeast S. cerevisiae using five different leader sequences' *see the whole article* ---	1-21
	-/--	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : 'A' document defining the general state of the art which is not considered to be of particular relevance 'E' earlier document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. '&' document member of the same patent family		
Date of the actual completion of the international search 23 June 1995		Date of mailing of the international search report - 5. 07. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer Marie, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL, vol. 12, no. 1, 1993 pages 285-294, Y. BOURBONNAIS ET AL. 'Isolation and characterisation of S. cerevisiae mutants defective in somatostatin expression...' *see the whole article* -----	1-21
Y	YEAST, vol. 6, 1990 pages 127-137, M. EGEL-MITANI ET AL. 'A novel aspartyl protease allowing KEX2-independent MF alfa propheromone processing in yeast' *see the whole article* -----	1-21

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